The human liver glutathione S-transferase gene superfamily: expression and chromosome mapping of an H_b subunit cDNA

Jeff L.DeJong[1,2], Chi-Ming Chang[2], Jacqueline Whang-Peng[3], Turid Knutsen[3] and Chen-Pei D.Tu[1,2]*

[1]Department of Molecular and Cell Biology, Pennsylvania State University, University Park, PA 16802, USA, [2]Institute of Molecular Biology, Academia Sinica, Taipei 11529, China and [3]Medicine Branch, NCI, NIH, Bethesda, MD 20892, USA

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ABSTRACT

We have isolated from a λgt10 cDNA library a clone λGTH4 which encodes a human liver glutathione S-transferase H_b subunit, designated as subunit 4. Expression of this cDNA in E. coli and subsequent purification and immunoblotting analysis provided a definitive assignment of a structure and function relationship. RNA blot hybridization with human liver poly(A) RNA revealed a single band of ~1200 nucleotides, comparable in size to the rat brain Y_{b3} mRNA. Divergence analysis of amino acid replacement sites in subunit 4 relative to the four rat Y_b subunits revealed that it is most closely related to the brain-specific Y_{b3} subunit. This conclusion is further substantiated by the nucleotide sequence homology between λGTH4 and the Y_{b3} cDNA in their 3' untranslated region. In situ chromosome mapping has located this glutathione S-transferase gene in the region of p31 on chromosome 1. Results from many laboratories, including ours, indicate that the human glutathione S-transferases are encoded by a gene superfamily which is located on at least two different chromosomes.

INTRODUCTION

The GSH S-transferases (GST, EC 2.5.1.18) are a family of dimeric proteins that are multifunctional in drug biotransformation and xenobiotics metabolism. High multiplicity of GST isozymes with overlapping substrate specificities is required to detoxify a multitude of xenobiotics in addition to serving other important physiological functions, such as protection against peroxidative damage (for a recent review, see ref. 1).

Recently, additional functions have been implicated for GSTs. Bennett et al. provided evidence that the nonhistone protein BA, previously demonstrated to co-localize with U-snRNPs within discrete nuclear domains, is a GST (2). The parasitic helminths of the genus Schistosoma have surface antigens that are GSTs. Acquired immunity in mice, rats, hamsters, and monkeys against this antigen from S. japonicum or S. mansoni has been shown to mediate significant protection against Schistosomiasis, a chronic debilitating disease in several parts of the world (3-5).

Multiple GSTs have been purified from human liver cytosol (1,6-13) and

extrahepatic tissues (1,13-15). Genetic polymorphism of GST expression has been observed and confirmed at the GST_1 locus, one of the three better characterized loci, which encodes some of the near neutral human GSTs (15-19), reported earlier as H_b (μ) class subunit(s) (10,11). We have previously established the sequence and immunological relationship between the human H_a and rat Y_a subunits (11,20-21). The relationship between the H_b (μ) and Y_b subunits has not been thoroughly investigated, however. In this communication, we report on the characterization of an H_b class cDNA by sequencing, RNA and DNA blot analysis, and expression in E. coli, as well as its chromosomal localization by in situ hybridization.

MATERIALS AND METHODS

Materials

Nucleotides, dNTPs and ddNTPs, and the plasmid expression vector pKK223-3 were obtained from Pharmacia L.K.B. Biotechnologies Inc. (Milwaukee, WI). [α-^{32}P] dCTP (sp. act. 3200 Ci/mmol) was purchased from New England Nuclear (Boston, MA), ICN (Irvine, CA) or Amersham (Arlington Heights, IL). [α-^{35}S] dATP was purchased from Amersham. Restriction endonucleases, T_4 DNA ligase, DNA polymerase I and the Klenow fragment were purchased from New England Biolabs (Beverly, MA) and Boehringer Mannheim (Indianapolis, IN). GSH, S-hexyl GSH, S-hexyl GSH agarose, p-nitro tetrazolium blue, 5-bromo-4-chloro-3-indolyl phosphate (X-gal), isopropylthiogalactoside (IPTG), tetracycline, chloramphenicol, ampicillin, streptomycin, 1-chloro-2,4-dinitrobenzene (CDNB), goat anti-rabbit IgG-alkaline phosphatase conjugated antibodies, and rat liver GSTs were all purchased from Sigma (St. Louis, MO). The human liver λgt11 cDNA library was a generous gift of Professor Harry Harris and Mitchell J. Weiss of the University of Pennsylvania. Another library in λgt10 was constructed from the same poly(A) RNA preparation by Dr. Gregory Grove in our lab. Both of the cDNA libraries and the poly(A) RNA for expression analysis were prepared from a single male human liver sample (11). The DNA for the genomic blot hybridization was prepared from a human placenta. The nick translation kit was purchased from BRL (Gaithersburg, MD). The GENECLEAN kit for DNA fragment isolation was purchased from Bio101 (San Diego, CA). Purified human liver GSTs and antisera raised against them were prepared as described previously (11).

DNA sequencing and other manipulations

Phage DNA preparation, subcloning, library screening with DNA probes, restriction enzyme digestions, and gel electrophoresis were all performed

essentially as described in Maniatis et al. (22). DNA sequence analysis was performed by the dideoxy chain-termination method (23) with [α-^{35}S]dATP (24) and M13 mp18 and M13 mp19 subclones (25). RNA blot hybridizations with rat and human liver poly(A) RNAs were carried out as previously described (11,28). DNA sequence homology was performed using the Dot Matrix Program of Zweig (26). Divergence calculations were done with DIVERGE by Perler et al (27). The programs were run on an IBM PC-AT.

Expression of the pGTH4 cDNA

pKK223-3 based clones containing the EcoRI insert in the correct orientation in E. coli JM105 were designated as pGTH4-KK, clones of opposite orientation were designated as pGTH4-KK'. An overnight culture was diluted 1:100 into LB media (50 ml) containing ampicillin (100 μg/ml) and streptomycin (25 μg/ml) at 37°C. IPTG was added to a final concentration of 2 mM when the A_{600} was between 0.3 and 0.4. Cells were allowed to grow for another 6-8 hours and were harvested by centrifugation. After resuspension in 10 mM KPi (pH 6.8), the cells were sonicated three times with a Branson sonifier (model 450) at setting 2 for 15 sec each. The debris was pelleted and the supernatant was assayed for activity against CDNB (29).

Large scale preparations (8 liters) were processed similiarly, and were passed through an S-hexyl glutathione affinity column (2 ml volume) after dialysis. The column was washed with 0.2 M KCl, 25 mM Tris-HCl pH 8.0 until the A_{280} was less than 0.05. The protein was eluted with 5 mM S-hexyl glutathione and 2.5 mM glutathione in the same buffer, and then dialyzed against 10 mM KPi (pH 6.8) and 0.2 mM DTT. The purified protein was analyzed on SDS-13.5% polyacrylamide gels (30). Western blotting was performed after electroblotting as described (31). The immunological cross reaction was observed by alkaline-phosphatase-conjugated anti-rabbit IgG and p-nitro tetrazolium blue (31).

In situ human chromosome mapping

High resolution metaphase chromosomes of lymphocytes from females were prepared using the FUDR-synchronized method. The in situ hybridization technique was performed according to Harper and Saunders (32). The pGTH4 cDNA insert was nick-translated with [^{3}H]TTP to a specific activity of 2 x 10^{7} cpm/μg.

RESULTS AND DISCUSSION

Isolation and characterization of pGTH4

We have screened a human liver λgt11 cDNA library using the cDNA insert

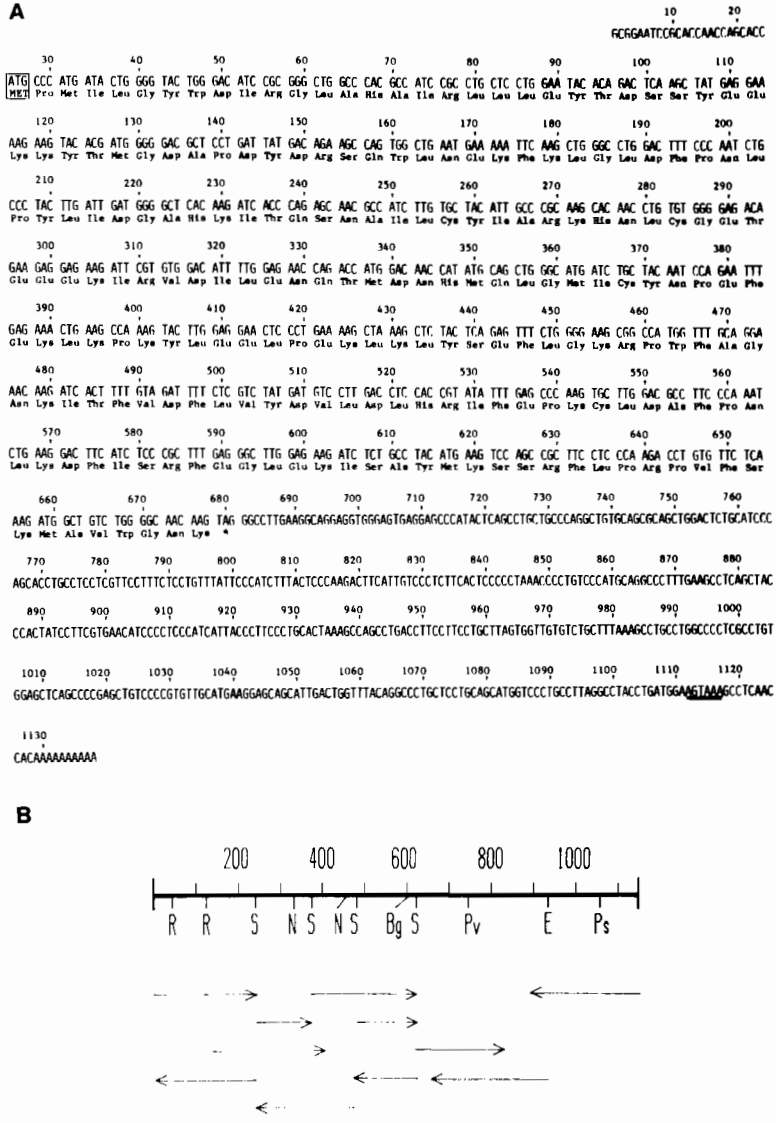

Figure 1. Characterization of the human liver H_b subunit cDNA clones, λGTH4A and λGTH4. A. Nucleotide and deduced amino acid sequence. The ATG initiation codon is boxed, and the termination codon is marked by an asterisk. The putative poly(A) addition signal is doubly underlined. The sequence is numbered one through 1130, is marked at every 10th position and includes 10 nucleotides of poly A. B. Partial restriction map and sequencing strategy. Arrows and lines represent the direction and length of the sequence determinations. The figure is a composite of the λGTH4A and λGTH4 clones (see text). The nucleotide at position 928 is where the 3' end of λGTH4A terminates. Bg, BglII; E, EcoRI; N, NcoI; R, RsaI; S, Sau3A; Ps, PstI.

from the rat Y_{b2} clone pGTR187 (28) to isolate H_b subunit cDNA(s). This approach was taken when screening with antisera (33) against total human liver GST was not successful (see discussion). From one of the libraries screened we isolated a clone, λGTH4A. Its cDNA subclone in pUC18 was designated as pGTH4A. After sequence analysis, we found that this cDNA has an apparent 3' truncation. A second λgt10 library was screened with the EcoRI insert of pGTH4A. A longer cDNA clone, λGTH4 was isolated and its pUC18 subclone was designated as pGTH4. The cDNA in λGTH4A is 9 nucleotides longer in the 5' untranslated region while that of λGTH4 contains the entire 3' untranslated region, including the poly(A) tail. These clones are otherwise identical as determined by DNA sequence analysis. The results, including sequencing strategy and partial restriction map, are shown in Fig. 1. The nucleotide at position 928 is the point at which the 3' untranslated sequence ends in λGTH4A.

The composite cDNA sequence is 1128 base pairs long not including the 10 nucleotides of A residues (Fig. 1). The 5' and 3' untranslated regions are 24 and 450 nucleotides long, respectively. A putative poly(A) addition signal (AGTAAA) is present between nucleotides 1112 and 1117 and is 12 nucleotides upstream of the A residues. The longest open reading frame spans nucleotides 25 through 678 and encodes a protein of 217 amino acids, excluding the N-terminal methionine (Fig. 1). The calculated molecular weight and pI of the deduced amino acid sequence are 25,561 and 6.6, respectively, establishing it

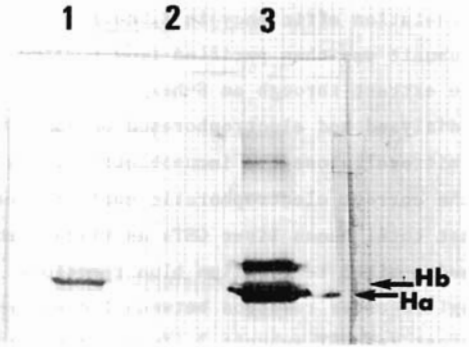

Figure 2. Western blot analysis of the affinity column-purified H_b subunit expressed in E. coli. The antisera used was against total human liver GSTs. Lane 1, total rat liver GSTs (~0.5 μg); lane 2, E. coli expressed H_b protein (~1 μg); and lane 3, total human liver GSTs (~1 μg) as purified by S-hexyl GSH affinity chromatography (11).

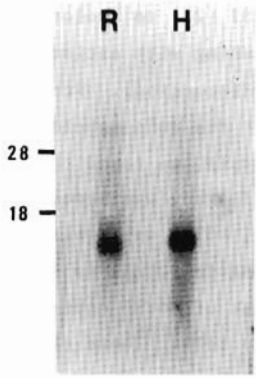

Figure 3. RNA blot hybridization of rat (R) and human (H) liver poly(A) RNAs (~10 µg). The EcoRI insert of pGTH4 was used as the probe. The numbers to the left indicate mobility of the 28S and 18S rRNA markers.

as a member of the H_b (µ) class of GSTs. We designate this sequence as human GST subunit 4.

Expression of pGTH4 cDNA sequence in E. coli

Because of its shorter 5' untranslated region, the EcoRI insert of λGTH4 was subcloned into the expression vector pKK223-3 in E. coli JM105. Pilot cultures (50 ml) of pGTH4-KK, induced with IPTG, were sonicated after concentration and assayed for transferase activity against CDNB. The relatively low activity we observed (0.7 µmoles/min/ml of bacterial culture) may be due to sequences in the 5' untranslated region which are less than optimal for high translation efficiency in E. coli.

The H_b-sized subunit was then purified from a large scale culture by passage of the crude extract through an S-hexyl GSH affinity column. The eluted protein was dialyzed and electrophoresed on an SDS-polyacrylamide gel and transferred to nitrocellulose for immunoblotting. As shown in Fig. 2, polypeptides with the correct electrophoretic mobility reacted immunologically with antisera against total human liver GSTs as visualized by the alkaline phosphatase catalyzed p-nitro tetrazolium blue reaction. There was no detectable immunological cross reaction between the expressed protein and antisera raised against rat GST subunit 3 (Y_{b1}), however (data not shown).

RNA and DNA blot hybridization

Approximately 10 µg of either rat or human liver poly(A) RNA was electrophoresed on a formaldehyde 1.5% agarose gel and transferred to a nylon filter. The entire EcoRI insert of λGTH4 was used as the probe with the results shown

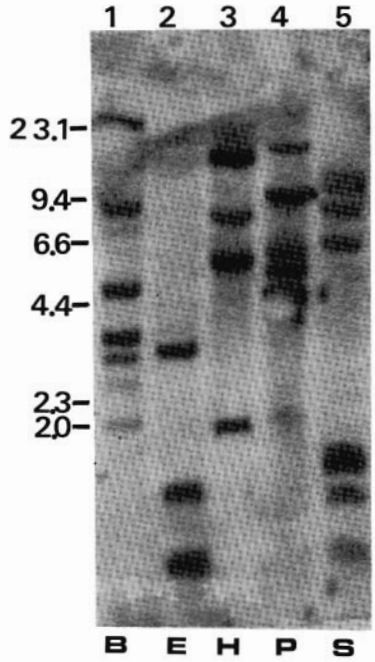

Figure 4. DNA genomic blot analysis of DNA from a human placenta. Approximately 10 µg of digested DNA was applied to each lane and transferred to a nylon membrane for hybridization with the EcoRI insert of pGTH4 as the probe. Lane 1, BglII (B); lane 2, EcoRI (E), lane 3, HindIII (H); lane 4, PstI (P); and lane 5, SstI (S). Numbers to the side are the sizes (kb) of λ DNA digested with HindIII.

in Fig. 3. As expected, this probe detects a band of approximately 1100 nucleotides in the rat liver poly(A) RNA. This represents hybridization of the λGTH4 coding sequence to the Y_{b1} and Y_{b2} messenger RNAs in rat liver. In the human liver poly(A) RNA a single hybridizing band of ~1200 nucleotides is seen. No message of a size comparable to those found in the rat liver is detected in this particular human liver RNA preparation.

Genomic DNA blot analysis (Figure 4) was performed with genomic DNA prepared from a single human placenta using the λGTH4 EcoRI insert as the probe. The presence of multiple bands of varying intensity in each lane suggests the existence of a multigene family.

Chromosome in situ hybridization

To determine the chromosomal location of the pGTH4 cDNA sequence, in situ hybridization was performed. Figure 5 shows the distribution of grains

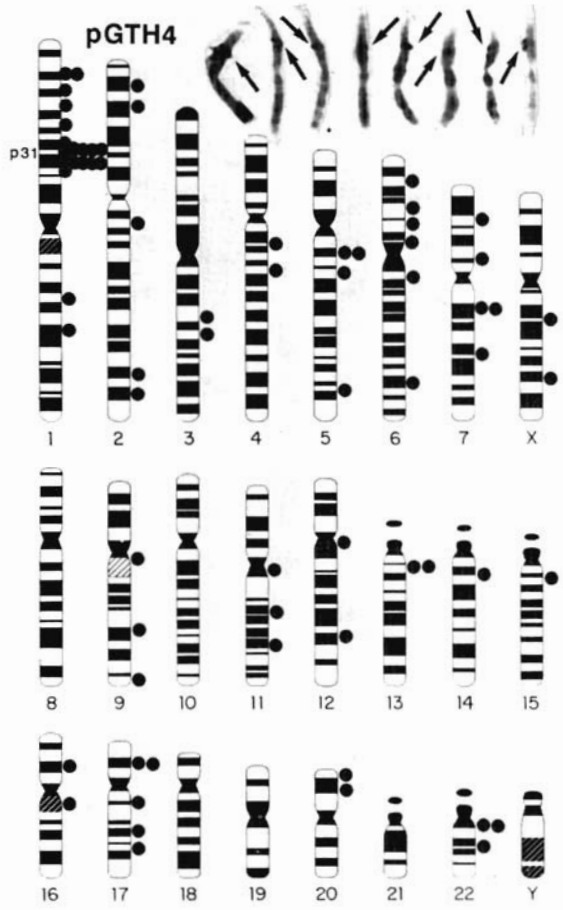

Figure 5. Schematic representation of FUDR-synchronized Wright-stained metaphase chromosomes indicating all chromosomal grains observed in 40 metaphases; 12 of the total 69 grains (17.4%) were located at 1p31. At the upper right corner of the Figure is shown 8 individual number 1 chromosomes from 8 different metaphases. Arrows indicate the grain (grains) at the 1p31 region.

observed over all the designated chromosomes: 17 of the 69 grains from 40 metaphases were located on chromosome 1p (a cluster of grains from the same area equals one grain), with 70% of the grains being observed in the region of 1p31. These data suggest that the pGTH4 gene is located at 1p31.

Comparison with the rat Y_b sequences

We have compared the deduced amino acid sequence of our pGTH4 cDNA to

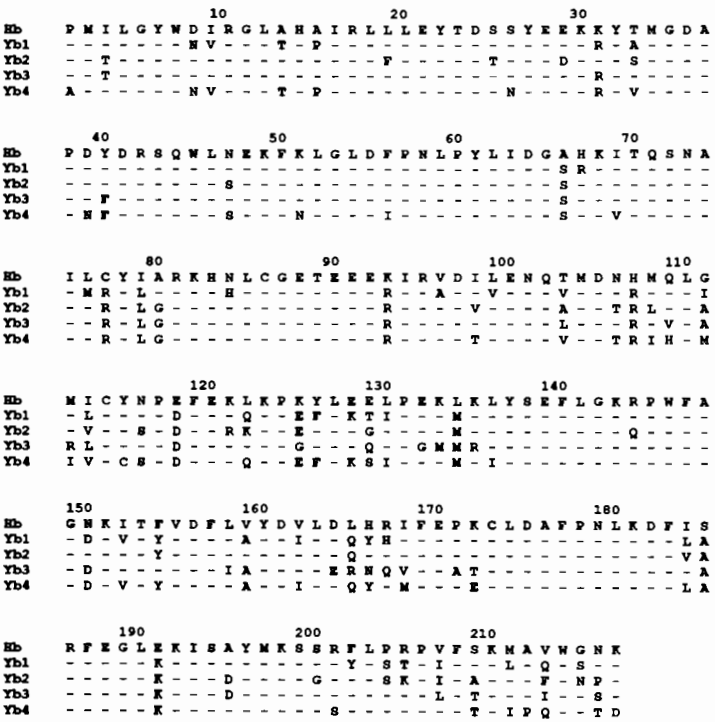

Figure 6. Amino acid sequence comparison between human GST subunit 4 and rat Y_{b1} (34), Y_{b2} (28), Y_{b3} (35,36), and Y_{b4} (37) subunits. The coding sequences of the four cDNAs and one genomic-clone-derived sequence (Y_{b4}) are designated by the single letter amino acid code and are aligned vertically. The first residue shown is the one following the initiating methionine. Dashes in the Y_b's represent amino acids identical to those in the H_b subunit 4 (top line).

those of the rat Y_{b1} (34), Y_{b2} (28), Y_{b3} (35-36), and from a genomic-clone-deduced sequence Y_{b4} (37) (Fig. 6). The percentage amino acid substitutions between the human subunit 4 sequence and the rat Y_b's was lowest for the Y_{b3} (18% amino acid differences, 38/217) followed by Y_{b2} (19%, 40/217), Y_{b1} (21%, 45/217), and Y_{b4} (27%, 58/217). Only 6% (12/217) of the amino acid substitutions between Y_{b3} and subunit 4 are not of conserved polarity. Also, the amino acid substitutions are not evenly distributed throughout the proteins. Clusters of changes occur from amino acids 103 to 136, 150 to 172, and from 200 to 217 (the C-terminus).

The close relationship between subunit 4 and the Y_{b3} subunit is further supported by the analysis of amino acid replacement sites (Table 1). The

Table 1. Divergence for Amino Acid Replacement Sites in Human and Rat GSTs

Pairwise comparison	% Divergence replacement sites	Divergence time (Myr) replacement sites
H_b (pGTH4) - Y_{b1} (pGTR200)	11.3	128.4
H_b (pGTH4) - Y_{b2} (pGTR187)	10.0	113.6
H_b (pGTH4) - Y_{b3} (pGTR301)	9.27	105.4
H_b (pGTH4) - Y_{b4} (λGTR15-2)	14.4	163.6
H_a (pGTH1) - Y_a (pGTR261)	14.7	167.0
H_a (pGTH1) - Y_c (pGTR302)	15.1	171.6

percentage divergence at replacement sites (9.7%) is lowest among all the pairwise comparisons. In addition, dot matrix analysis revealed nucleotide sequence homology in the 3' untranslated regions of these two DNA sequences (Fig. 7). This somewhat scattered homology extends from nucleotides 780 to 910 of the pGTH4 clone, and from 770 to 900 of the Y_{b3} clone.

DISCUSSION

We have characterized a new cDNA clone encoding subunit 4 (H_b or μ class) of the human liver GSTs. The deduced amino acid sequence contains 217 amino acids with a calculated molecular weight of 25,561. To provide a more definitive structure-function relationship between the cDNA clone and its protein, we have expressed this cDNA clone in *E. coli*. The low activity against CDNB observed is most probably due to the suboptimal length in the 5' untranslated region for efficient translation in *E. coli*. Trimming of the 5' untranslated region to reduce the distance between the ribosome binding site on pKK223-3 and the ATG initiation codon of the cDNA may increase our expression level. Nevertheless, activity levels in the crude extract were above background (i.e., pGTH4-KK' in JM105). In addition, we have analyzed the expressed, affinity column-purified protein by SDS-polyacrylamide gel electrophoresis and immunoblotting. Our human liver GST antisera specifically recognize a protein of the expected H_b subunit mobility. Therefore, we have independently demonstrated that the clone λGTH4 encodes a human liver GST H_b subunit, designated as subunit 4. The weakness of the immunological reaction provides a possible explanation for our failure to isolate the H_b subunit cDNA

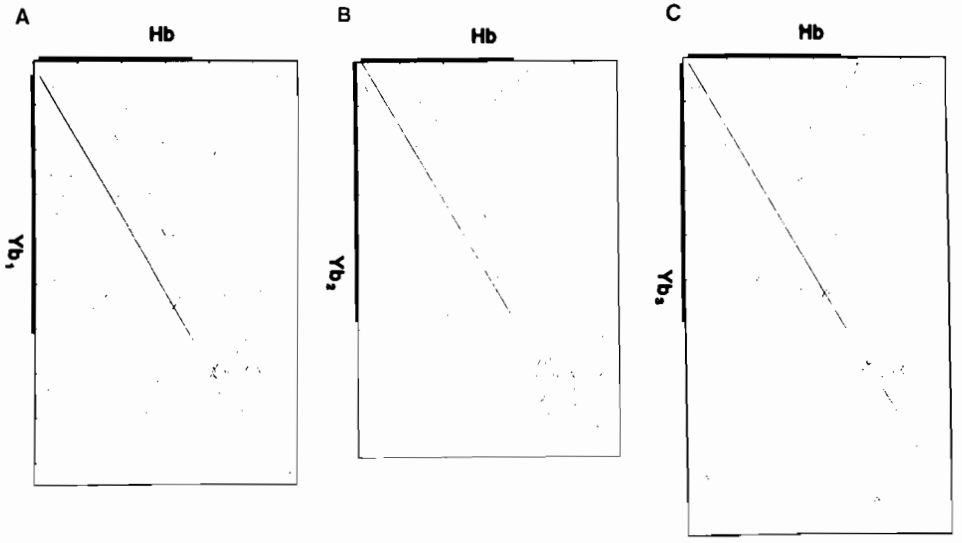

Figure 7. Comparison of the H_b and various rat Y_b nucleotide sequences by the dot matrix analysis method. H_b is always shown on the horizontal axis. The vertical axis represents A) Y_{b1}, pGTR200 (34); B) Y_{b2}, pGTR187C (28,37); and C) Y_{b3}, pGTR301 (35,36). The stringency parameter was 20 out of a 25 nucleotide window. Solid bars represent amino acid coding regions with the remainder being either the 5' or 3' untranslated regions.

clones with the antibody screening procedure (33).

Our analysis on the expression of this H_b gene in a male human liver revealed the existence of a single hybridizing mRNA band. This band migrated as a species larger than the rat liver mRNAs, Y_{b1} and Y_{b2}. This is consistent with both the length of our λGTH4 cDNA clone, and with the length of Y_{b1} and Y_{b2} cDNA clones published earlier (28,34). This size variation is due to their different lengths in the 3' untranslated regions. This result also suggests that other possible H_b class and related mRNAs, if expressed in this individual, should be of ~1.2 kb in length. Our repeated failure in isolating additional H_b cDNA clones, however, suggests that this may be the only H_b mRNA in the liver of this individual as can be detected by these methods. Consistent with this is the fact that isoelectric focusing gels of affinity purified GST protein from the liver of this individual show a single near-neutral migrating band (data not shown). The genomic DNA blotting result indicates the presence of at least several homologous genes or pseudogenes. Characterization of genomic clones should identify the nature of the H_b gene family organization.

Of the 38 amino acid changes in subunit 4 relative to the rat Y_{b3} sequence, only 12 residues are not of similar polarity. This is consistent with the lowest percentage divergence (9.7%) at replacement sites among all the H_b-Y_b pairwise comparisons (Table 1). If we assume that the 3' untranslated region has been free of selective pressure, the sequence conservation in this region (Fig. 6) convincingly demonstrates that the gene encoding human subunit 4 is orthologous to the rat Y_{b3} gene sequence, whose expression is predominantly in rat brains (35,36,38).

The percent divergence among all the H_b-Y_b pairwise comparisons is considerably lower than the pairwise comparisons of H_a-Y_a or H_a-Y_c (Table 1 and refs. 20,21). The reason why the H_b-Y_b family of GSTs has diverged less is not clear at present.

GST zymograms from a biochemical genetic study involving liver samples from 179 individuals from three racial groups have been reported (16). It was suggested that a common null allele exists at the GST_1 locus (encoding H_b or μ class subunits) at a relatively high frequency in the Caucasian, Chinese, and Indian populations studied. Analysis of affinity chromatography-purified liver GSTs from several individuals revealed the presence of multiple H_b (μ) class subunits in some and the absence of them in others (10,19). It is possible that our male caucasian liver sample is either homozygous for subunit 4 or a heterozygote containing one null allele on one chromosome 1. We also know that this same individual has two different H_a genes at 6p12 encoding subunits 1 and 2 (20,21,39). Thus, the H_a and H_b subunits are products encoded by two different gene families located on two different chromosomes.

There is a detectable level of nucleotide sequence homology between λGTH4 and λGTH1 (H_a) cDNAs only within regions encoding amino acids 63 to 97 of the H_b subunit. This region contains 45% amino acid sequence identity relative to the H_a subunit, thus suggesting functional conservation for GST activities. The human GST-II deduced amino acid sequence (40), upon optimal alignment with the H_b sequence, shows approximately 28% amino acid identity. Given the sequence similarities between these three classes of human GSTs, and also considering the many reports on the purification of multiple GSTs in man (6-19), it is most probable that human GSTs are products of a gene superfamily, similar to the genes encoding rat GSTs.

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*To whom correspondence should be addressed

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